Chapter 1
Introduction
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Introduction and Review of Literature

1.1. Bacterial Surface Layers (S-layers): An introduction

Most prokaryotic organisms (bacteria and archaea) possess a well defined supramolecular cell-wall structure outside the plasma membrane (Beveridge and Graham, 1991), which presumably evolved in the course of evolution. The presence of this periodic structure containing a single protein or glycoprotein monolayer was first discovered in electron micrographs of bacteria belonging to the genus Spirillum in 1953 (Sleytr, 1978). Although these crystalline, subunit type surface layers (S-layers) were still considered rare and unusual in microorganisms, similar structures were found on the surface of many other bacteria using advanced electron microscopic techniques (Sleytr and Messner, 1983). As of today, S-layers have now been identified in every taxonomic group of eubacteria (walled bacteria) and represent an almost universal feature in archaeabacteria (Sleytr et al., 1996; Messner and Sleytr, 1992).

The S-layer is the outermost component of the bacterial cell envelope, overlying the cell wall and completely covering the cell (Fig. 1.1). S-layers are composed of single protein or glycoprotein species forming two dimensional crystalline arrays on the cell surface. S-layer subunits have an inherent ability to self assemble into two dimensional arrays on the supporting envelope layer, and display defined symmetry and uniform pore size (Hovmoller et al., 1988; Sleytr and Messner, 1983).
Due to their location on the cell surface, S-layers are directly involved in the interactions of cell with its surrounding environment. Upon prolonged cultivation on laboratory media bacteria lost the ability to synthesize S-layers, indicating their possible role in conferring survival advantage to cell in different habitats (Sleytr and Messner, 1983).

1.2. Occurrence and distribution of S-layers

Bacterial S-layers represent a unique set of biological structures. They represent the only wall-component outside the plasma membrane and frequently appear integrated into the lipid layer (Baumeister et al., 1990; Baumeister and Lembcke, 1992). In Gram-positive bacteria and in archaea, the S-layer lattice is attached to a rigid wall matrix, which is composed of peptidoglycan or pseudomurein respectively (Fig. 1.2) (Konig, 1988; König and Messner, 1997). Monomolecular arrays of S-layer proteins have also been observed in bacterial sheaths (Beveridge and Graham, 1991) and on the surface of the cell wall of eukaryotic algae (Roberts et al., 1982). Till date, S-layers have been described for 600 microbial species and 10 % of all characterized bacteria and archaea (Sleytr, 1978; Messner and Sleytr, 1992; Beveridge and Graham, 1991; Sara and Sleytr, 2000).
S-layer is even considered as one of the simplest biological membranes to be optimized during biological evolution. S-layer producing organisms are ubiquitous in biosphere; many species of bacteria and most archaea possess a crystalline S-layer as the outermost component of their cell envelopes. These structures were thought to be evolved due to a selective response to specific environmental and ecological pressures. Despite a considerable diversity, two-dimensional proteinaceous arrays of S-layers remain one of the most commonly observed features of prokaryotic cell envelope structures (Sleytr, 1978; Sleytr et al., 1988).

1.3. Detection of S-layers

At present, there are no simple techniques available for detecting the presence of S-layer protein on the cell surface. Preliminary screening procedures involve analysis of the crude preparation of membrane proteins by SDS-PAGE. The presence of a high molecular weight protein band in high concentration is indicative of an S-layer (Priest, 1992). But this method is error prone since many bacteria belonging to the *Bacillus* species are known to
produce high molecular weight non-S-layer proteins in very high concentrations.

The S-layer arrays on cell surface can be unequivocally identified by transmission electron microscopy (TEM). Transmission electron microscopy (TEM) procedures such as freeze-etching, thin sectioning, freeze-drying in combination with heavy metal shadowing (Fig. 1.3) and negative staining are the best methods to identify S-layer arrays on the cell surface (Sleytr and Messner, 1992; Beveridge, 1981, 1994). Deep etching of freeze-fractured replicates of shock frozen cell suspension has been routinely used for detection and calculation of the extent of surface coverage by S-layer (Sleytr et al., 1969; Holt et al., 1969). Etching of cell surface will expose the subunit profile because the grains of the shadowing metal such as platinum are relatively large (~ 0.2-0.5 nm). Only the general form of alignment of the S-layer subunits is revealed by freeze-etching (Sleytr and Messner, 1983 and 1988). On the contrary, negative staining with a heavy metal salt such as phosphotungstate and uranyl acetate enshrouds S-layer subunits revealing contours of proteinaceous components and offers better resolution (Beveridge et al., 1994; Pum et al., 1989). Since this method requires dehydration with organic solvents, native protein conformation can be altered (Sidhu and Osten, 1997).

![Fig. 1.3. Electron micrograph of a freeze-etched and Pt-C-shadowed preparation of a Gram-positive organism exhibiting a S-layer lattice. (Sleytr et al., 1986)](image)
Although TEM analysis using different cell treatment protocols provides a visual evidence for the presence of S-layer, the complete chemical and ultrastructural characterization of the S-layer protein requires a purified sample. Hence, isolation of the S-layer from the cell surface becomes mandatory.

1.4. Isolation of S-layers

S-layer lattices of different bacteria differ considerably in their susceptibility to isolation and disassembly into monomeric subunits from the cell envelope structure. In other words, they exhibit a variable susceptibility to the treatment of different denaturing agents. Hence, a variety of strategies have been designed by considering the nature of intra- and inter-subunit bonding. A general method for S-layer isolation from cell wall fragments involves mechanical cell disruption, using different approaches such as French press, sonication, cell homogenization with glass beads or freeze thawing. Subsequently, separation and purification of the S-layer from the cell fragments is achieved by differential centrifugation (Sleytr, 1978; Thorne, 1977).

In Gram-positive organisms, cells are initially treated with lysozyme (Sleytr, 1978; Thorne, 1977) and the adherent cytoplasmic membrane fragments are selectively solubilized by addition of Triton X-100 (Breitwieser et al., 1992; Beveridge, 1979; Sleytr and Glauert, 1976). Since, S-layer subunits are bonded by non-covalent forces with each other and with the cell envelope structure; lysozyme-treated cells are treated with low concentrations of chaotropic agents, e.g., 0.5 M urea, 1-2 M guanidine hydrochloride, to loosen the bonds with the supporting polymer, without affecting the lattice of the S-layer (Henry, 1972; Nermut and Murray, 1967; Sleytr and Messner, 1983).

Alternatively, S-layer lattices are completely disintegrated by treating cells or cell walls with high concentrations of hydrogen bond-breaking agents such as urea, guanidine hydrochloride (Sara and Sleytr, 1994, Kuen et al., 1994, Sleytr et al., 1986), by addition of detergents such as sodium dodecyl
sulphate (SDS) (Kotiranta et al., 1995), by decreasing or increasing the pH of the solution (Sleytr, 1976) or by cation substitution (e.g., replacing Ca$^{2+}$ by Na$^+$ or Li$^+$ ions) (Messner and Sleytr, 1992; Sleytr et al., 2001). Removal of the denaturing agent by dialysis allows the S-layer to reassemble into two-dimensional crystalline lattices, identical to those observed in intact cells (Jaenicke et al., 1985; Sleytr et al., 2001).

In case of Gram-negative organisms, disintegration of S-layers from cell wall into its monomeric subunits have been achieved by treating with metal chelating agents such as ethylenediaminetetraacetic acid (EDTA), ethylenbis(oxymethylenenitrilo)tetraacetic acid (EGTA) (Beveridge and Murray, 1976; Sleytr, 1981; Sleytr et al., 1996; Beveridge, 1994), by addition of low concentrations of urea or guanidine hydrochloride (Buckmire and Murray, 1970; Hastie and Brinton, 1979; Thornley et al., 1974), SDS (Bauminster, 1982; Koval and Murray, 1983) or lithium 3,5-diiodosalicylate (Koval and Murray, 1983), by cation substitution using acid, alkali (Beveridge and Murray, 1976) or detergents (Koval and Murray, 1983; Winter et al., 1978) or by using combinations of these techniques (Thornley et al., 1974; Evenberg and Lugtenberg, 1982; Kay et al., 1981).

Special isolation and purification procedures are applied to Archaebacteria, where the S-layer is attached to the plasma membrane (e.g., Sulfolobus acidocaldarius, Halobacterium sp.). Treatments with Triton X-100 and SDS, changes in pH and ionic strength of the solution and organic solvent extraction have been used for the isolation of S-layer in case of Archaebacteria. However, surface layers from some archaea are very resistant to extraction and disintegration, suggesting the presence of covalent inter-subunit bonding (Beveridge, 1994; Schultze-Lam and Beveridge, 1994; Sleytr and Messner, 1983; Sleytr et al., 1999).

In many cases, these proteins are detected by transmission electron microscopy, so little information is obtained about their properties to permit completely reliable selection of an isolation and purification technique. Therefore, some experimentation may be necessary to find an appropriate
procedure for a bacterial strain not examined previously, or for a newly discovered protein (Messner and Sleytr, 1988).

The confirmation and structural investigation of the S-layer isolated and purified from Gram-positive bacteria, Gram-negative bacteria or Archaeabacteria is carried out using TEM and Atomic Force Microscopy (AFM) analysis.

1.5. Recrystallization of S-layer proteins and confirmation of structure

With the development of high resolution microscopic techniques such as High Resolution Transmission Electron Microscopy (HR-TEM), Scanning Tunneling Microscopy (STM) and Atomic Force Microscopy (AFM), detection and characterization of S-layer lattices with nanometer resolution has become possible (Fig. 1.4) (Dufrene, 2001a and b; Muller et al., 1999; Scheuring et al., 2002). Purified subunits of S-layer were allowed to recrystallize on a suitable substrate (depending upon the instrument for detection) and visualized using TEM and AFM.

![Fig. 1.4. High-resolution AFM topographs of S-layer of Corynebacterium glutamicum recorded in buffer solution (10 mM Tris-HCl, pH 7.5, 250 mM KCl) lead to identification of the flower-shaped surface. (Scheuring et al., 2002).](image)
However, recrystallization conditions vary for S-layers isolated from different strains, and therefore, need to be optimized for each strain independently (Sleytr et al., 2003).

1.6. Chemical composition of S-layer

Different combinations of weak bonds (hydrophobic bonds, ionic bonds involving divalent cations or direct interaction of polar groups and hydrogen bonds) are responsible for the structural integrity of S-layers (Sleytr and Messner, 1983). The subunits are linked together and also linked to the underlying cell envelope layers by non-covalent forces. In some archaeobacteria, S-layers are the only cell wall structure and therefore are associated with the plasma membrane. (Messner and Sleytr, 1992; Beveridge and Graham, 1991; Konig, 1988). Moreover, the secondary cell wall polymers and not the peptidoglycan, serve as anchoring site for many S-layer proteins (Ries et al., 1997; Lemaire et al., 1995, 1998; Ilk et al., 1999; Chauvanx et al., 1999).

SDS-PAGE and Matrix-Assisted Laser Desorption and Ionization-Time of Flight (MALDI-TOF) analysis of isolated S-layers showed the presence of identical proteins or glycoproteins of molecular mass 30-220 kDa (Sleytr et al., 1994; Messner and Sleytr, 1992). In fact, S-layers are the first prokaryotic glycoproteins reported in the archa and bacteria. Thus, the assumption that bacteria cannot covalently cross link glycan chains to their cellular protein was disproved (Schaffer and Messner, 2001). Many bacteria possess S-layers composed of more than one type of subunit, as revealed by SDS-PAGE analysis (Abe et al., 1983; Tsuboi et al., 1982).

Comparative studies of amino acid residues revealed a similarity in overall composition of S-layer proteins in organisms from different phyla (Beveridge, 1994; Sara and Sleytr, 1996; Sleytr and Messner, 1983; Sleytr et al., 1996). Although a high content of acidic and hydrophobic amino acids have been detected, a very low content of arginine, histidine and methionine.
is present. On the other hand, lysine is found to be predominant basic amino acid (Messner, 1996; Sara and Sleytr, 1996a and b).

Most S-layer proteins are composed of weakly acidic proteins having an isoelectric point in the range 4-6. Exceptions are the S-layers proteins of different lactobacilli (Boot and Pouwels, 1996; Sara and Sleytr, 2000) and Methanothermus fervidus (Brockel et al., 1991), which possess isoelectric points in the range of 9-11.

In the S-layer array, chemical functional groups are not only aligned in high density but also present at identical position and orientation on each protomer (Fig. 1.6). In different members of Bacillaceae, the distribution and functional significance of charged groups on S-layer proteins have shown that free amino (-NH$_2$) and carboxyl (-COOH) groups on adjacent protomers are arranged in close proximity. It indicates that S-layer lattice are stabilized by electrostatic interactions between the charged groups (Sara and Sleytr, 1993).

![Fig. 1.6](image.jpg)

**Fig. 1.6.** Diagrammatic representation of morphological features and distribution of chemical groups on S-layer array. The yellow circles represent the functional group arranged on the S-layer backbone (red).
Among S-layers isolated from different members of Bacillaceae, the electric charge on the inner and outer surface is significantly different although there are no significant differences in the amino acid composition (Sara and Sleytr, 2000, Sleytr et al., 2001a and b). The charge on the outer surface is neutral due to an equimolar proportion of exposed amino and carboxyl groups, whereas, the inner surface can possess either a net positive or net negative charge (Sara and Sleytr, 1993; Sleytr et al., 2001a and b; Sara and Sleytr, 2000; Boot and Pouwels, 1996).

1.7. S-layer homology and variation

S-layer genes from many archaea and bacteria have been cloned and sequenced (Bowditch et al., 1989; Sara and Sleytr, 2000; Sleytr et al., 1999). By comparison, sequence similarities have been identified at the N-terminal part of many S-layer proteins and are designated as S-layer homologous motifs (SLH). In Gram-positive bacteria, SLH motifs have been identified at the N-terminal part, which are involved in anchoring of S-layer to the cell surface via specific molecular interactions (Olabarria et al., 1996; Ilk et al., 1999; Etienne-Toumelin et al., 1995; Sleytr et al., 2001). However, low sequence similarities have been detected in the middle and C-terminal part (Leibovitz et al., 1997; Ebisu et al., 1990; Sara and Sleytr, 2000).

In S-layer producing bacteria having an average generation time of 20 min, approximately 500 subunits of S-layer need to be produced per second and incorporate into the existing S-layer array (Sleytr et al., 1983). Therefore, promoters of bacterial S-layer genes must be very strong to keep surface of cell covered with S-layer (Kuen and Lubitz, 1994). According to literature, S-layer promoter of Lactobacillus acidophilus is considered to be twice as efficient as LDH (Lactate dehydrogenase), which is one of the strongest known promoters in bacteria (Boot et al., 1995, 1996). The presence of multiple promoters for S-layer gene and their differential expression has been established in case of B. stearothermophilus ATCC 12980 and B. brevis (Adachi et al., 1989; Scholz et al., 1997; Jarosch et al., 2000).
There is also increasing evidence about the expression of alternative S-layer protein genes by S-layer-carrying bacteria for adaptation to different stress factors, such as the immune response of the host for pathogens and drastic changes in the environmental conditions for non-pathogens. Variations of the S-layer proteins have been described in detail on the molecular level for Campylobacter fetus (Blaser et al., 1994). Changes in the S-layer protein have been described in Campylobacter fetus (Garcia et al., 1995; Dworkin and Blaser, 1996), Lactobacillus acidophilus ATCC 4356 (Boot et al., 1996), Bacillus stearothermophilus (Sara and Sleytr, 1994; Sara et al., 1996b) and Thermus thermophilus HB8 (Olabarriya et al., 1996) in response to various environmental factors. In Campylobacter fetus, silent S-layer gene cassettes are involved in S-layer protein variation (Dworkin et al., 1995). In addition, silent S-layer gene copies have been identified in Bacillus sphaericus and Lactobacillus acidophilus (Boot et al., 1995). In most of the characterized cases, the mechanism of S-layer variation is based on DNA rearrangements (Boot et al., 1996; Dworkin and Blaser, 1996; Sara and Sleytr, 2000; Scholz et al., 2001; Jakava-Viljanen et al., 2002).

1.8. Lattice Characteristics and \textit{in vitro} self-assembly of S-Layer proteins

The protein or glycoprotein subunits of S-layers are endowed with the ability to assemble into two dimensional arrays on the supporting cell envelope component of prokaryotic organisms. High resolution transmission electron microscopical and scanning force microscopic studies have shown that pores in the porous sheets of S-layer occupy up to 70 % of their surface area (Sleytr et al., 1999). An average sized procaryotic cell is covered with $\sim 5 \times 10^5$ S-layer subunits. Thus, to maintain the S-layer lattice on its surface, the cell synthesizes $\sim 500$ copies per second (Sleytr and Messner, 1983; Sleytr et al., 1999; Sara and Sleytr, 2000).

Since, S-layers are monomolecular assemblies of identical subunits; they exhibit pores of identical size and morphology (Hovmoller et al., 1988; Sleytr
and Messner, 1983). Thus, their structures can be classified according to their space groups, unit cell size, and position of protomers and pores, which give rise to a peculiar symmetric pattern. S-layers lattices can be aligned in unit cells of hexagonal (p3, p6), square (p4), or oblique (p1, p2) symmetries (Sara et al., 1996a and b; Egelseer et al., 1995; Beveridge, 1994, 1981; Beveridge and Koval, 1993; Hovmoller et al., 1988; Koval, 1988; Sleytr and Messner, 1988a, b, 1983; Baumeister and Engelhardt, 1987; Koval and Murray, 1986). Depending upon the lattice type, one morphological unit of oblique, square and hexagonal lattice consists of two, three, four and six monomers, respectively (Fig. 1.7).

The centre-to-centre spacing of the morphological units can range from 3 to 35 nm (Sleytr et al., 1994, 2001b; Sara and Sleytr, 2000). Most S-layer arrays have a smooth outer surface and a corrugated inner surface with a thickness of 5-15 nm.

![Fig. 1.7. Lattice symmetries of S-layer unit cells (Sleytr et al., 2001)](image)
A remarkable feature of isolated S-layer subunits of Gram-positive and Gram-negative bacteria is their ability to reassemble into monomolecular crystalline arrays with the same lattice dimensions as those observed in intact cells (Sleytr and Messner, 1983; Sleytr et al., 2001b). Isolated S-layer subunits can recrystallize on the cell envelope fragments from which they have been removed, on the cell envelope fragments of other organisms or even on untextured charged or uncharged inanimate objects such as glass, polymers, silicon wafers, mica etc. (Pum et al., 1993; Pum and Sleytr, 1994, 1995a, 1995b; Sleytr and Messner, 1992; Sleytr et al., 2001b, 2003).

The self-assembly mechanism is mainly determined by the morphology and bonding properties of S-layers. In solution, S-layers can form open-ended cylinders, flat sheets or closed vesicles, which are possible only in the case of hexagonal lattice symmetries of S-layers (Messner and Sleytr, 1992; Sleytr and Messner, 1983, Sleytr et al., 2001b). Additionally, recrystallization of S-layers is influenced by physical and chemical parameters such as temperature, pH, ionic strength, ion composition and bivalent cations. A slight change in these parameters induces different assembly processes and lead to morphological changes (Sleytr and Messner, 1983; Sleytr et al., 2003; Toca-Herrera, 2004).

During the self-assembly process, the assembly of precursor molecules is rapid in the initial phase and is followed by a slower phase of recrystallization, leading to formation of large-area arrays (Toca-Herrera et al., 2004; Jaenicke et al., 1985; Sleytr et al., 2003). Moreover, the process of assembly starts at various nucleation points at the same time and grows in a plane until neighboring growing areas meet each other. The process is also dependent upon the number of nucleation sites and the amount of protomers available per site for incorporation into the array (Pum et al., 1993; Pum and Sleytr, 1994; Sleytr et al., 2001b, 2003).

The total calcium concentration plays a significant role in the process of recrystallization. The absence of calcium results in a deformed structure or an
incomplete array covering short patches only (Sleytr et al., 2001a and b; Toca-Herrera, 2004).

Thus, the S-layer assembly into an array is an entropy-driven process and totally dependent upon the amino acid sequence of polypeptide chains without the need for any morphological template (Sleytr, 1978; Sleytr et al., 2003). Further, only selected S-layer proteins show a capability to reassemble into large coherent monolayers on different solid supports, at the air/water interface and on lipid films, as shown in Fig. 1.8 (Sleytr et al., 2001b).

Its inherent ability to self-assemble in an array combined with its inherent physicochemical and lattice structural properties on the nanometer scale makes S-layers a unique and natural organic structure. Thus, S-layers can be considered as an ideal matrix for functionalization of surfaces (Sidhu and Oslen, 1997) and have led to a broad spectrum of applications (Sleytr et al., 1997).

Fig. 1.8. Diagrammatic representation of the recrystallization of isolated S-layer subunits into arrays (Sleytr et al., 2001).
1.9. Biological functions of S-layers

Very little information is available about the specific biofunctions of S-layers. From a very general point of view, these metabolically expensive products may provide the organism with a selective advantage in very diverse habitats. S-layers completely cover the cell surface and can confer selective advantage by functioning as protective coats, molecule or ion traps (Sleytr and Messner, 1983; Sidhu and Osl, 1997, Sara and Sleytr, 2000; Sleytr et al., 2001b; Beveridge, 1997) and molecular sieves in the ultrafiltration range (Sara and Sleytr, 1987a and b). S-layers can also play an important role in cell adhesion and surface recognition and provide a frame work for determining the cell shape in Gram-negative archaea (Hovmoller et al., 1988; Baumeister et al., 1990; Beveridge, 1994, Sara and Sleytr, 2000).

S-layers can play a major role as virulence factors in pathogenic bacteria (Beveridge, 1994; Sleytr et al., 1988a; Sleytr and Messner, 1992, 1988a, b, 1983; Beveridge and Graham, 1991; Hovmoller et al., 1988; Koval, 1988; Baumeister and Engelhardt, 1987). In case of Aeromonas salmonicida, its S-layers endows organism with high resistance against bactericidal activity of complements in immune or non-immune sera. S-layer exhibit unique property of binding to immunoglobulin as well as extracellular matrix protein binding ability and plays an important role in uptake of porphyrins (Doig et al., 1992; Garduno et al., 1995).

S-layers of Bacillus species act as adhesion sites for cell associated exoenzymes. In Staphylothermus marinus membrane anchored stalks held S-layer glycoprotein at 70 nm distance from plasma membrane. Two proteases stable at hyperthermal temperature are found to be associated with each stalk (Mayr et al., 1996; Peters et al., 1995). Interestingly, binding of such high molecular weight proteins does not affect the diffusion of metabolites or nutrients through the S-layer lattice, as observed in case of B. steaortermophilus (Egelseer et al., 1995; Mayr et al., 1996; Egelseer et al., 1996). In case of B. sphaericus, they provide adhesion sites for bacteriophage (Howard and Tipper, 1973).
S-layers are also known to protect cells from attack of proteolytic enzymes (Buckmire and Murray, 1973). S-layers of Gram-negative bacteria such as *A. salmonicida*, *C. fetus*, *A. serpens* and *C. crescentus* function as protective coats by conferring resistance against attack of bacterial parasites such as *Bdellovibrio bacteriovorus*. However, they can not perform same function against other predators like protozoa (Koval, 1997). In another interesting protective function S-layers of *Synechococcus* functions as template for fine-grain mineralization which is continuously shed from the cell surface to prevent clogging of cell envelop layers (Schultze-Lam and Beveridge, 1994). The S-layer of *B. cereus*, commonly found in periodontal infections, was found to confer a survival advantage to the organism against attack of polymorphonuclear leukocytes (Kotiranta et al., 1995 and 1997).

In *Rickettsia prowazekii* and *R. typhi*, the S-layer is responsible for humoral and cell mediated immunity (Carl and Dasch, 1989). S-layer of *Lactobacillus acidophilus* plays an important role in adhesion of cells to the intestinal epithelium (Schneitz et al., 1992).

### 1.10. Applications of S-layers

Due to their uniform pore sizes and periodically arranged functional groups, S-layers have broad application potential in the fields of molecular nanotechnology, nanobiotechnology and biomimetics (Sleytr et al., 1997, 2001). The following sections describe the applications of S-layers in different areas:

#### 1.10.1. Immobilization of molecules

S-layer is considered as an ideal matrix for immobilization of molecules because the functional groups on the surface are arranged in a well-defined position and orientation. This precise alignment of functional groups results in a systematic binding of functional molecules on the surface. For
immobilization of biologically active macromolecules, carboxylic acid groups on the S-layer can be activated by treatment with cross linking molecules such as 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC). The activated carboxylic acid groups can subsequently react with the free amine groups present on enzymes, antibodies or ligands such as streptavidin or protein A (Sara and Sleytr, 1989) (Fig. 1.9). The carbohydrate chains present in the glycosylated S-layer protein can also be exploited for immobilization of macromolecules (Kupcu et al., 1996; Sleytr and Sara, 1997).

Fig. 1.9. Schematic depiction of S-layer as matrix for immobilization of biomolecules (Sleytr et al., 2007).

1.10.2. Isolation and purification of biomolecules

The unique property of S-layers to form arrays of uniform pore size has been utilized for preparation of S-layer ultrafiltration membranes (SUM). Ultrafiltration membranes created by deposition of S-layer self-assembly products on microporous supports offer an alternative to traditional synthetic polymer-based ultrafiltration membranes. S-layer ultrafiltration membranes were used for purification of enzymes and it was observed that molecular exclusion limit is a species specific characteristic (Sara and Sleytr, 1987a and b; 2000) (Fig. 1.10).
Affinity microparticles coated with S-layer have been successfully used for isolation of IgG from cell culture supernatants or serum (Figure 1.11).

These microparticles do not induce cytokine and showed no cytotoxicity when used for extracorporeal blood purification (Weiner et al., 1994; Weber et al., 2000; Kucu et al., 1996). Breitwieser et al. (1998) demonstrated the use of S-layer ultra-filtration membranes in the development of dipstick-style solid-phase immunoassays.
1.10.3. Development of biosensors

S-layer lattices have been used as immobilization matrices for a broad range of macromolecules in the development of amperometric and optical biosensors. In these devices, enzymes are covalently linked to the self-assembly products of S-layer protein. For example, in a glucose sensor to monitor blood glucose levels, glucose oxidase is covalently bound to the surface of SUM. To establish the electrical contact between sensing layer and equipment, a thin film of gold (20-50 nm) is sputter coated on the enzyme immobilized surface. The analyte percolates through the porous-SUM and reaches to the sensing layer. During the process, the enzymatic reaction converts glucose to hydrogen peroxide, which is directly proportional to the concentration of glucose. The glucose concentration is measured by measuring the current of the electrochemical oxidation of hydrogen peroxide. SUM has also been used for multienzyme sensors, such as sucrose sensors with three enzyme species (invertase-mutarotase-glucose oxidase). Further, SUM has been used for development of optical biosensors using fluorescent dye (Ruthenium-II-complex) for glucose sensing (Neubauer et al., 1993, 1994, 1996; Taga et al., 1994).

1.10.4. Immunotherapy

With the increased understanding of S-layer genetics, recombinant and genetically modified S-layer (glycol) proteins have been synthesized and used successfully as carrier/adjuvants for vaccination and immunotherapy (Sleytr, 1997; Sleytr et al., 2001b). Since reproducible immobilization of ligands to currently used carrier molecules cannot be achieved, S-layer self assembly products were tested as adjuvants and immune enhancer. Various strategies have been deployed for development of S-layer vaccine. It is known that S-layer of Aeromonas salmonicida and Aeromonas hydrophila are essential for virulence. These are pathogens of fish and cause furunculosis leading to significant economic losses. Numerous strategies have been established for vaccination of salmon or trout against furunculosis using S-layer preparations (Udey and Fryer, 1978; Ford and Thune, 1992; Kay et al., 1981).
1.10.5. Nanopatterning

In 1986, Douglas et al. reported for the first time an approach for the development of perfectly ordered array of nanoparticles using S-layer as a template. S-layer fragments of *Sulfolobus acidocaldarius* were coated with ~1 nm thick tantalum/tungsten film, which on subsequent ion milling formed a structure of 15 nm pores arranged in a hexagonal symmetry. Subsequently, wet chemical processes for patterning of nanoparticles using S-layer as a template have been developed (Hall et al., 2001). In this approach, monolayers of S-layers are exposed to a metal salt solution, followed by a slow reaction with a reducing agent. Monolayers of S-layer attached to different substrates such as glass and silicon wafer have been used to induce the formation and alignment of cadmium sulfide as shown in Fig. 1.12 (Shenton et al., 1997) and gold nanoparticles (Dieluweit et al., 1998). In these experiments, inorganic superlattices were formed using particles of 3–5 nm diameter, in either oblique or square lattice symmetry and an inter-particle spacing of ~10-13 nm.

![Fig. 1.12. Transmission Electron Micrograph of CdS nanoparticles patterned by biomineralization of S-layer (Scale bar 60 nm) (Sleytr et al., 1997)](image-url)
Two-dimensional arrays of S-layer have also been explored as masks for deep UV patterning (DUP) by Pum et al. (1999) (Fig. 1.13).

Fig. 1.13. Schematic illustration of patterning of S-layers with deep ultraviolet radiation.

(a) A pattern is transferred onto the S-layer through a microlithographic mask that is brought into direct contact with the S-layer.

(b) The S-layer is specifically removed from the silicon surface at the exposed regions but retains its structural and functional integrity in the unexposed areas.

(c) The unexposed regions can be used for selectively binding biologically functional molecules or reinforced for subsequent reactive-ion etching.

(Pum and Sleytr, 1999)
Traditional techniques used for patterning such as photolithography (lithography using a UV light source), X-ray lithography (lithography using short wavelength X-Rays), e-beam lithography (lithography using electronic beam), ion-beam lithography, nano-imprint lithography, scanning probe microscopy and self-assembled monolayers (ordered crystalline monolayers of alkanethiols, oxides and carboxylic acids formed by adsorption onto a suitable surface) offer the following advantages:

1. Ability to build hard structures on micron scale lengths with sub-μm features (lithography and scanning probe microscopy).
2. Control of chemical properties at the molecular level (self-assembled monolayers).
3. No requirements of masks (lithography).
4. Computer-controlled (lithography and scanning probe microscopy) and fast process (X-ray lithography).

However, all lithographic techniques have inherent drawbacks such as non-deposition of molecule-based nanostructures directly on a conducting surface (Purn and Sleytr, 1999), highly equipment- and cost-intensive, and require reliable ion/X-ray sources. On the other hand, formation of self-assembled monolayers require costly chemicals (alkanethiols cost $500 per 5 mg) and annihilation of functional residues combined with changes in wetting and transport properties of the surface causes loss of function (Haruyama et al., 2005). Lastly, all techniques are surface-specific and hence cannot be used universally.

In comparison, monomolecular arrays of S-layer subunits bind to solid surface by hydrophobic interactions. Thus, complicated surface modifications for adhesion promotion and crystal growth are not required. Another advantage of using S-layer as masks is that non-specific attachment of molecules such as proteins can be minimized. Hence, S-layers offer an alternative material for nanopatterning and can open up new avenues to study molecular interactions and generate patterned arrays of nano- and macromolecules.
1.11. Definition of the problem

In recent years interest in fabrication of sub-nanometer (1 μm-100nm) and nanometer structures have increased tremendously. The rise of nanotechnology has raised hopes of achieving goals which were unrealistic some time ago and nanotechnology is shaping up well as science of 21st century. From fundamental point of view, nanostructures are useful tools to study properties such as electrical, magnetic, optical, thermal, and mechanical at the nanometer level, whereas from applied aspect, nanostructures are of prime importance today because they offer significant improvement in the existing electrical, optical devices and technologies. As electronic devices are approaching lower limits, the importance of nanoparticles as molecules has considerably increased. This is due to wide spread application of nanoparticles ranging from biomedicine to microelectronics and in defense too. The realization of the full potential of nanotechnological systems, however, has so far been limited due to the difficulties in their synthesis and subsequent assembly into useful functional structures and devices. Development of defect free monolayers for patterning organic-inorganic interface in the nanometer range without losing the structural and functional integrity of the matrix are the key prerequisites for it.

There are two major principles of nanotechnological construction which are being used currently (a) top-down, which involves microscopic manipulation with a few atoms or molecules and generation of an ordered pattern of such structures, and (b) bottom-up, which involves the parallel self-assembly of molecules. Despite all the promise of science and technology at the nanoscale, the control of nanostructures and ordered assemblies of materials in two- and three-dimensions still remains largely elusive with the current state-of-the-art, top-down techniques such as lithography. Therefore bottom-up, specifically bio-inspired synthesis of nanomaterials is gaining prominence due to the fact that these techniques are less demanding in terms of equipments, time and cost as compared to top-down processes. In addition, biological processes are not energy or equipment intensive and do not require toxic chemicals, thus minimizing toxic waste products.
Thus biological structures capable of self-assembly are expected to play a central part in the bottom-up approach. In essence, nanobiotechnology takes advantage of inherent property of self-organization and molecular recognition of natural biopolymers such as proteins and nucleic acids. Self-assembly of protein subunits is a commonly observed phenomenon in nature e.g. virus capsids, bacteriophages, pili, bacterial flagella, etc. However, very little is known about surface layers (S-layers), which are ordered two-dimensional protein structures involved in the envelope of some bacteria and archaea. Unique properties of S-layers described earlier make them ideal candidates for functionalization of surfaces which can be exploited for molecular patterning. Self assembly products of S-layers would be suitable alternate approach to existing methods for synthesis of metal nanoparticles of broad size range (3-15 nm in diameter) with constant interparticle spacing on S-layers template.

Although the presence of S-layer has been reported in more than 200 strains of bacteria and archaea, S-layers from many of these bacteria are not suitable for technological applications, primarily due to the pathogenic nature of the culture or difficulty in obtaining biomass due to stringent growth requirements. Therefore it becomes mandatory to screen for new cultures which are non-pathogenic, easy to cultivate in laboratory conditions and capable of synthesizing S-layers, to fulfill the ever increasing demand of novel templates with unique morphology and properties.

Bacteria tend to lose S-layers when they are cultivated on laboratory media and unavailability of simple and easy to perform rapid detection tests limits the pace of research on this important topic. Moreover, research in this area is highly equipments-intensive requiring costly instruments such as High Resolution Transmission Electron Microscope (HR-TEM), Atomic force microscope (AFM), ultracentrifuge, etc. which limits the progress on this topic. Considering the wide application potential of S-layers and its technological significance, a majority of research in this frontline area is patented and may not be easily available in public domain. Therefore, it becomes imperative to
develop and standardize different methods in-house rather than relying on
published literature.

Although patterning of nanoparticles has been demonstrated using S-layer
template, it is still a largely unexplored field in the area of nanotechnology.
Unavailability of simple and easy procedures for synthesis and patterning of
nanoparticles on S-layer template is, once again one of the limiting factors.

Considering above background the present work was carried out with
following objectives:

1. Standardization of methods for the isolation, purification and
characterization of S-layers from *Bacillus* strains
2. Development of a method for easy and rapid detection as well
as purification of S-layers
3. Exploring possible application of S-layers as a template for
synthesis and patterning of gold nanoparticles